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Use of prolonged treatment and the fluctuation test to detect mutations in human fibroblasts treated with methyl methanesulfonate

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Summary

The methyl methanesulfonate (MMS) induction of 6-thioguanine-resistant ($6TG^r$) mutants in non-synchronized human fibroblasts has been quantitatively characterized. A 24-h MMS treatment failed to induce mutations in non-synchronized cultures. However, exposure of growing cells to MMS for 3 days caused a significant increase in the mutation frequencies. The mutation induction was dose-dependent. The fluctuation test was also useful in detecting the mutagenic activity of MMS in human fibroblasts. Prolonged treatment of cultures made the human cell mutagenesis testing system more sensitive.

Systems for detecting mutations in human cells have been developed (Albertini and DeMars, 1973; Jacobs and DeMars, 1977). Jacobs and DeMars (1978) employed *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to work out system details. Use of this system, however, has been limited to testing potent mutagens (Maher and Wessel, 1975; Huang and Lieberman, 1978; Jacobs and DeMars, 1978). We have repeatedly failed to induce 6TG-resistant cells after MMS treatment in non-synchronized human fibroblast using the conventional test procedures of Jacobs and DeMars (1978).

Recently, the Luria–Delbrück fluctuation test has been used to detect the effects of low concentration of mutagens or weak mutagens (Voogd et al., 1974; Cole et al. 1976; Green et al., 1976). It has been suggested that the fluctuation test is a

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simpler and more sensitive assay for mutagen detection than the conventional testing method (Cole et al., 1976). We have used the fluctuation test to demonstrate the mutagenic activity of MMS in human cells.

This paper describes the effects of prolonged treatment of MMS on the induction of mutations in non-synchronized human fibroblasts, and the use of Luria–Delbrück analysis to determine the rate of mutant induction.

Materials and methods

Cell lines, cultures, media and cell cloning. Human fibroblast lines were derived from neonatal foreskins. Cells were grown in 85 vol. of Ham's F10 medium (without hypoxanthine) and 15 vol. of fetal calf serum (FCS) with 100 units penicillin per ml and 0.1 mg kanomycin per ml at 37°C in a 5% CO_2 atmosphere. 6-Thioguanine selective medium (FCS-F10-TG) consisted of FCS–F10 and 7.5×10^{-5} M 6TG.

Cloning efficiency was measured by inoculating 100 cells into a 60-mm petri dish, renewing the medium after 1 week, and staining the cells at the second week with 2% crystal violet. Prior to staining, cultures were rinsed with saline and fixed with 10% formaldehyde.

Estimation of mutation frequency for prolonged treatments. 1.5×10^6 cells were plated on 100-mm dishes for 24 h and then treated with daily renewal of fresh MMS contained in fresh culture medium for a total treatment time of 24, 48 or 72 h respectively.

After different periods of chemical treatment, each dish of cells was washed twice with F10 medium. The cells were then cultures in FCS–F10 medium for 7 days with 2 passages. After 7 day expression, cells were plated in dishes to detect mutants and estimate cloning efficiency. The mutation frequencies were determined according to the method previously described (Jacobs and DeMars, 1977, 1978). 10^4 cells were plated per 60-mm dish; 120 dishes were prepared for each dose. All the dishes received 6TG at a final concentration of 7.5×10^{-5} M in 3 h. The selective medium was renewed once every week. For determination of cloning efficiency, 100 cells per 60-mm dish were plated and grown in FCS–F10 medium. Dishes for estimation of cloning efficiency were stained at the end of the second week and mutagenesis dishes at the end of the third week. Mutant colonies were counted and the mutation frequencies were determined according to the Poisson expectation (Albertini and DeMars, 1973; Maher and Wessel, 1975; Jacobs and DeMars, 1977, 1978; Huang and Lieberman, 1978).

Estimation of mutation rates using Luria–Delbrück fluctuation analysis. 3000 cells per dish were inoculated in 40 dishes and allowed to grow in the presence of MMS for 5 days with daily renewal of MMS. Cell number (N_t) from each dish was determined 7–9 days after inoculation and 10^4 cells from each dish were transferred to new dishes. All the new dishes received 6TG at a final concentration of 7.5×10^{-5}

M on the same day. The selective medium for these cultures were renewed once every week. For determination of cloning efficiency of these cells, 100 cells per 60-mm dish were plated and grown in FCS–F10 medium. Dishes for estimation of cell cloning efficiency were stained at the end of the second week and those for selection of mutants at the end of the third week.

Mutation events were assumed to be distributed among the culture dishes according to a random (Poisson) distribution. The average number of mutants per dish, X, was estimated from the fraction of dishes having no mutant clones, P_o. The negative log of P_o gave a mean number of mutants per dish. The average number of cell generations per dish, $\bar{N}$, was obtained according to the Luria and Delbrück formula $\bar{N} = (N_t - N_o)/\ln 2$ where N_o is the number of cells inoculated before MMS treatment and N_t is the number of cells at the onset of selection. N_o and N_t were corrected for cloning efficiency for calculation of mutation rates. The mutation rate (a) was calculated using the Luria and Delbrück formula: $a = X(\ln 2)/(N_t - N_o)$.

TABLE 1

CALCULATION OF INDUCED MUTATION FREQUENCIES AFTER REPEATED TREATMENT WITH MMS

The average number of mutant colonies (X) per dish was obtained according to $P_o = e^{-X}$, where P_o was the fraction of dishes bearing no mutants. Multiplication of the initial number of cells (1.1×10^4) per dish by the cloning efficiency of the cells gave the number of surviving cells per dish. Mutation frequencies were calculated by dividing X by the number of surviving cells per dish. Induced mutation frequencies were obtained by subtracting the background mutation frequency from the calculated mutation frequencies.

Period of treatment (h)	Concn. (μg/ml)	Cloning effic. (%)	P_o	Number of mutants per dish	Number of viable cells per dish	Mutation freq. ($\times 10^{-6}$)	Induced mutation ($\times 10^{-6}$)
24	0	33.4	0.962	0.038	3674	10.47	–
	3	34.6	0.904	0.101	3806	26.56	16.09
	6	34.4	0.918	0.085	3784	22.50	12.03
	12	37.6	0.892	0.114	4136	27.59	17.12
48	0	33.8	0.973	0.028	3718	8.18	–
	3	34.0	0.900	0.105	3740	30.98	22.80
	6	35.2	0.909	0.095	3872	27.08	18.90
	12	38.8	0.836	0.179	4268	46.05	37.87
72	0	33.8	0.973	0.028	3718	8.18	–
	3	28.8	0.927	0.076	3168	26.22	18.04
	6	27.8	0.911	0.094	3058	33.64	25.46
	12	31.6	0.862	0.148	3476	46.85	38.67

Results

The effects of prolonged treatment on the induction of mutation frequencies in non-synchronized cultures. Detection of mutants was done 7 days after the last treatment. The estimation of mutation frequencies was based on the exact probability function for the Poisson random variable. Table 1 shows the gradual increase in mutation frequencies following repeated treatment with MMS. After a 3-day exposure with chemical, the increase in the mutation frequencies was detectable and a dose-mutation frequency relationship was demonstrated (Fig. 1).

Detection of mutation using Luria and Delbrück fluctuation analysis. To increase the duration of treatment beyond 3 days, we inoculated a small number of cells into culture dishes, exposed the cells to MMS for 5 days and grew them in MMS-free medium for 2–4 more days. The Luria and Delbrück formula was used to calculate mutation rates (20–40 dishes were used for each concentration). The actual initial

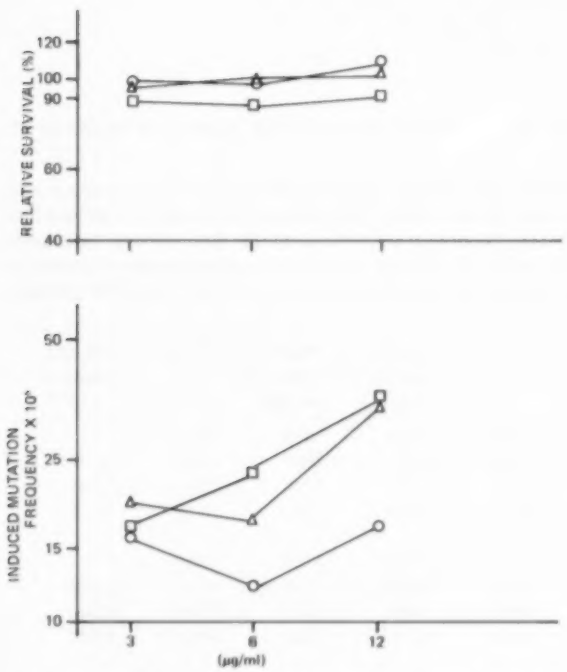

Fig. 1. Mutagenicity and cytotoxicity in non-synchronized human fibroblasts treated with MMS for 24 h (○), 48 h (△), or 72 h (□). After a 7-day expression, the cytotoxic effect of MMS diminished. The straight line fit for the induced mutation frequencies of cells treated for 72 h is given by: $\ln(y) = 2.29 + 0.55 \ln(x)$. Detailed data are presented in Table 2.

TABLE 2

MUTATION RATES FROM FLUCTUATION TEST FOR 6-THIOGUANINE RESISTANCE WITH MMS

The Luria and Delbrück fluctuation method was used to estimate mutation rate/cell/generation of treated and non-treated human fibroblasts. The initial number of cells averaged 887 per 60-mm dish (N_o). Cells were first grown in MMS for 5 days and then in MMS-free medium for 2–4 days. At the end of this period, the cell number/dish was determined (N_t) and the cells were transferred to new dishes for the detection of mutants. Their cloning efficiency was determined and N_t was corrected with this cloning efficiency. This new number (N_t) represented the actual surviving number of cells per dish. The per cell generation was calculated as $\bar{N} = (N_t - N_o)/\ln 2$. P_o was the proportion of dishes having no mutants. $1-P_o$ represents the proportion of dishes containing mutants. The negative log of P_o, $(-\ln P_o)$, gave a mean number of mutants per dish, X, which was then divided by the respective N, giving a mutation rate. Induced mutation rates were obtained by subtracting the respective spontaneous rates. Two mutation rates were determined for a, b, c and d groups on different dates. Both rates were similar.

Dose (μg/ml)	N_o	Cloning effic. (%)	N_t ($\times 10^4$)	$1-P_o$	Mean number of mutants per dish	Per cell per gener. ($\times 10^{-7}$)	Mutation rate ($\times 10^{-7}$)	Induced mutation rate
0	963	35.8	31.0	0.069	0.0715	15.90	4.50	–
3	963	30.5	32.0	0.143	0.1542	13.90	11.08	6.58
0[a]	738	27.9	7.4	0.060	0.062	2.89	21.46	–
0[a]	738	32.5	12.0	0.107	0.113	5.52	20.47	–
6[b]	738	12.9	12.0	0.156	0.169	2.13	79.19	58.22
6[b]	738	22.3	24.0	0.409	0.526	7.61	69.16	48.19
0[c]	960	32.0	12.0	0.024	0.024	5.54	4.33	–
0[c]	960	34.0	20.0	0.042	0.043	9.81	4.38	–
12[d]	960	7.3	7.1	0.056	0.057	0.61	94.18	89.83
12[d]	960	11.3	26.0	0.292	0.470	4.10	114.61	110.26

cell number per 60-mm culture dish averaged 887. No spontaneous mutants had been observed among 40 dishes in several earlier determinations. The results of several Luria and Delbrück fluctuation analyses are presented in Table 2. Spontaneous mutation rates were estimated to be 4×10^{-7} to 20×10^{-7}/cell/generation. Mutation rates of treated cultures increased significantly and induced mutation rates were linear with increasing concentrations of chemical (Fig. 2). As the result of prolonged treatment, all treated groups had increased the fraction $(1-P_o)$ of dishes carrying mutants. Rates were found consistent in duplicated measurements.

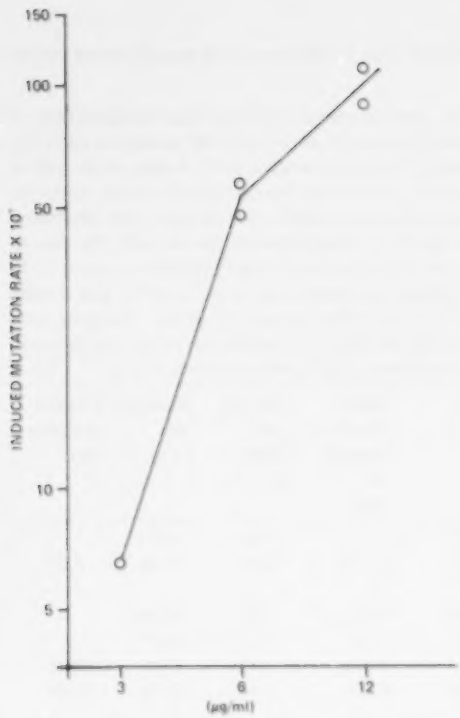

Fig. 2. Induced mutation rates of human fibroblasts treated with MMS for 5 consecutive days. The rates, per cell per generation, were obtained 7 and/or 9 days after the initiation of treatment using the Luria and Delbrück formula. Rates were consistent in duplicated measurements on different dates. The straight line fit for the induced mutation rates over the dose tested is given by: $\ln(y) = 0.11 + 1.91 \ln(x)$. Detailed data are presented in Table 2.

Discussion

MMS induces 6-thioguanine resistance in CHO cells (Couch et al., 1978), mutations at the TK locus in mouse lymphoma cells (Cole and Arlett, 1978) and sister-chromatid exchanges and chromosome aberrations in cultured cells (Palitti and Becchetti, 1977; Raff and Sugahara, 1980). However, MMS does not induce Oua-resistant variants in mouse lymphoma cells (Cole and Arlett, 1978). Using the fluctuation test, MMS mutagenic activity to Oua-resistance was demonstrated in mouse lymphoma cells (Cole et al., 1976). This was attributable to the effect of prolonged treatment (Cole et al. 1980). Similarly, MMS did not induce mutation in a conventional way (Jacobs and DeMars, 1977) in our experiments. However,

prolonged treatment with MMS in human cells resulted in a significant increase in mutation frequencies and a demonstrable dose-mutation frequency relationship. Using the Luria and Delbrück experimental design, we also demonstrate mutagenic activity of MMS in human cells.

Employing synchronized cell cultures (Huang et al., 1981) and using the conventional protocol (Jacobs and DeMars, 1977), we detected mutagenic activity of MMS in human cells (Huang et al., 1981). It has been reported that only S-phase cells were sensitive to mutagens, and inclusion of S-phase cells was necessary for induction of mutation (Taylor, 1960; Bader et al., 1963; Kikuchi and Sandberg, 1964; Mueller and Kajiwara, 1966; Mukherjee et al., 1968; Braun and Willis, 1969). This makes the use of synchronized cells rather sensitive, and explains why MMS failed to induce mutation in non-synchronized cells treated for short time periods.

Non-synchronized cultures contain various proportions of S-phase cells in their populations. Lenghtening treatment period would increase the opportunity of S-phase cells to be mutagenized and add new mutations to the cultures. This additive effect would enhance the mutation frequency. Using drug resistance as the marker for detection of mutation, one must consider the effect of phenotypic lag. 3–5 day expression has been shown sufficiently long enough to demonstrate dose-dependent induction of mutations (Mahar and Wessel, 1975; Huang and Lieberman, 1978; Jacobs and DeMars, 1978). This phenotypic lag did not present difficulties in fluctuation experiments. In fact, as the results indicated, 5 daily MMS treatments in the interval of 7–9 days growth period had produced a significant linear dose–response mutation.

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